NOVEL GENE AND METHOD FOR PRODUCING L-AMINO ACIDS

Technical field

The present invention relates to biotechnology, and more specifically to a method for producing amino acid, especially for a method for producing L-homoserine, L-threonine, L-valine or L-leucine using a bacterium belonging to the genus *Escherichia*.

Background Art.

The present inventors obtained, with respect to *E. coli* K-12, a mutant having mutation, *thrR* ~~(herein referred to as *rhtA23*)~~ that is concerned in resistance to high concentrations of threonine or homoserine in a minimal medium (Astaurova, O.B. et al., Appl. Bioch. And Microbiol., 21, 611-616 (1985)). The mutation improved the production of L-threonine (SU Patent No. 974817), homoserine and glutamate (Astaurova, O.B. et al., Appl. Bioch. And Microbiol., 27, 556-561, 1991) by the respective *E. coli* producing strains.

Furthermore, the present inventors has revealed that the thrR mutation ~~*rhtA* gene~~ exists at 18 min on *E. coli* chromosome and that the mutation arose in ~~*rhtA* gene~~ is identical to ORF1 between *pexB* and ompX genes. The unit expressing a protein encoded by the ORF has been designated as *rhtA* (rht: resistance to

homoserine and threonine) gene. The *rhtA* gene includes a 5'-noncoding region including SD sequence, ORF1 and a terminator. Also, the present inventors have found that a wild type rhtA gene participates in resistance to threonine and homoserine if cloned in a multicopy state and that the ~~rhtA23~~ thrR mutation is caused by an A-for-G substitution at position -1 with respect to the ATG start codon in the rhtA gene (the mutation was designated by "rhtA23") (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457).

It is found that at least two different genes which impart threonine and homoserine resistance in a multicopy state exist in *E. coli* during cloning of the *rhtA* gene. One of the genes is the *rhtA* gene, and the other gene was found to be *rhtB* gene which confers homoserine resistance (Russian Patent Application No. 98118425).

Disclosure of the Invention

An object of the present invention is to provide a a method for producing an amino acid, especially, L-homoserine, L-threonine and branched chain amino acids

with a higher yield.

The inventors have found that a region at 86 min on *E. coli* chromosome, when cloned by a multicopy vector, impart resistance to L-homoserine to cells of *E. coli*. the inventors further found that there exists in the upstream region another gene, rhtC, which involves resistance to threonine, and that when these genes are amplified, the amino acid productivity of *E. coli* can be improved like the *rhtA* gene. On the basis of these findings, the present invention have completed.

Thus, the present invention provides:

(1) A bacterium belonging to the genus *Escherihia*, wherein L-threonine resistance of the bacterium is enhanced by enhancing an activity of protein as defined in the following (A) or (B) in a cell of the bacterium:

(A) a protein which comprises the amino acid sequence of SEQ ID NO: 4; or

(B) a protein which comprises the amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence of SEQ ID NO: 4, and which has an activity of making a bacterium having the protein L-threonine-resistant;

(2) The bacterium according to (1), wherein L-

homoserine resistance of the bacterium is further enhanced by enhancing an activity of protein as defined in the following (C) or (D) in a cell of the bacterium:

(C) a protein which comprises the amino acid sequence of SEQ ID NO: 2; or

(D) a protein which comprises the amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence of SEQ ID NO: 2, and which has an activity of making a bacterium having the protein L-homoserine-resistant;

(3) The bacterium according to (1) or (2), wherein the activity of protein as defined in (A) or (B) is enhanced by transformation of the bacterium with DNA coding for the protein as defined in (A) or (B);

(4) The bacterium according to (2), wherein the activity of protein as defined in (C) or (D) is enhanced by transformation of the bacterium with DNA coding for the protein as defined in (C) or (D);

(5) A method for producing an amino acid, comprising the steps of:

cultivating the bacterium as defined in any one of (1) to (4), which has an ability to produce an amino acid, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid

from the medium;

(6) The method according to (5), wherein the amino acid is selected from the group consisting of L-homoserine, L-threonine and branched chain amino acids;

(7) The method according to (6), the branched chain amino acid is L-valine or L-leucine.

(8) A DNA which encode a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 4;

(B) a protein which has the amino acid sequence of SEQ ID NO: 4 including substitution, deletion, insertion, addition, or inversion of one or several amino acids, and has an activity of making a bacterium having the protein L-threonine-resistant.

9. The DNA of (8) which is a DNA defined in the following (a) or (b):

(a) a DNA which comprises the nucleotide sequence of nucleotide numbers 187 to 804 in SEQ ID NO: 3;

(b) a DNA which is hybridizable with a nucleotide sequence of nucleotide numbers 187 to 804 in SEQ ID NO: 3 or a probe prepared from the nucleotide sequence under a stringent condition, and encodes a protein having an activity of making a bacterium having the protein L-threonine-resistant; and

10. The DNA of (9) wherein the stringent condition is a condition in which washing is performed at 60 °C, and at a salt concentration corresponding to 1 x SSC and 0.1 % SDS.

The DNA fragment coding for the protein as defined in the above (A) or (B) may be referred to as "*rhtC* gene", a protein coded by the *rhtC* gene may be referred to as "*RhtC* protein", the DNA coding for the protein as defined in the above (C) or (D) may be referred to as "*rhtB* gene", a protein coded by the *rhtB* gene may be referred to as "*RhtB* protein". An activity of the RhtC protein which participate in resistance to L-threonine of a bacterium (i.e. an activity of marking a bacterium having the RhtC protein L-threonine-resistant) may be referred to as "Rt activity", and an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of marking a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity". A structural gene encoding the RhtC protein or RhtB protein in the *rhtC* gene or *rhtB* gene may be referred to as "*rhtC* structural gene" or "*rhtB* structural gene". The term "enhancing the Rt activity or the Rh activity" means imparting resistance to threonine or homoserine to a bacterium or enhance the resistance by means of

increasing the number of molecules of the RhtC protein or RhtB protein increasing a specific activity of these proteins, or desensitizing negative regulation against the expression or the activity of these proteins or the like. The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-threonine resistance means a property that a bacterium grows on a minimal medium containing L-threonine at a concentration at which a wild-type strain thereof not grow, usually at >30 mg/ml. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild-type strain thereof not grow, usually at >5 mg/ml. The ability to produce an amino acid means a property that a bacterium produce and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

According to the present invention, resistance to threonine, or threonine and homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. A bacterium belonging to the genus *Escherichia*, which has increasing resistance to threonine, or threonine and homoserine and an ability to accumulate an amino acid, especially, L-homoserine, L-

threonine, or branched chain amino acids such as L-valin and L-leucine in a medium with a high yield.

The present invention will be explained in detail below.

<1> DNA used for the present invention

The first DNA fragment used for the present invention (*rhtC* gene) coding for a protein having the Rt activity and having the amino acid sequence of SEQ ID NO: 4. Specifically, the DNA may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 187 to 804 of a nucleotide sequence of SEQ ID NO: 3.

The second DNA fragment used for the present invention (*rhtB* gene) coding for a protein having the Rh activity an having the amino acid sequence of SEQ ID NO: 2. Specifically, the DNA may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence of SEQ ID NO: 1.

The rhtB gene having the nucleotide sequence of SEQ ID NO: 1 corresponds to a part of sequence complement to the sequence of GenBank accession number M87049, and includes f138 (nucleotide numbers 61959-61543 of M87049) which is a known but function-unknown ORF (open reading frame) present at 86 min on *E. coli* chromosome, and 5′-

and 3'- flanking regions thereof. The f138, which had only 160 nucleotides in the 5'-flanking region, could not impart the resistance to homoserine. No termination codon is present between the 62160 and 61959 nucleotides of M87049 (upstream the ORF f138). Moreover, one of the ATG codons of this sequence is preceded by a ribosome-binding site (62171-62166 in M87049). Hence, the coding region is 201 bp longer. The larger ORF (nucleotide numbers 62160 to 61546 of M87049) is designated as *rhtB* gene.

The *rhtB* gene may be obtained, for example, by infecting Mucts lysogenic strain of *E. coli* using a lysate of a lysogenic strain of *E. coli* such as K12 or W3110 according to the method in which mini-Mu d5005 phagemid is used (Groisman, E.A., et al., J. Bacteriol., 168, 357-364 (1986)), and isolating phagemid DNAs from colonies growing on a minimal medium containing kanamycin (40 μg/ml) and L-homoserine (10 mg/ml). As illustrated in the Example described below, the *rhtB* gene was mapped at 86 min on the chromosome of *E. coli*. Therefore, the DNA fragment including the *rhtB* gene may be obtained from the chromosome of *E. coli* by colony hybridization or PCR (polymerase chain reaction, refer to White, T.J. et al, Trends Genet. 5, 185 (1989)) using oligonucleotide(s) which has a sequence corresponding to

the region near the portion of 86 min on the chromosome *E. coli*.

Alternatively, the oligonucleotide may be designed according to the nucleotide sequence of SEQ ID NO: 1. By using oligonucleotides having nucleotide sequences corresponding to an upstream region from the nucleotide number 557 and a downstream region from the nucleotide number 1171 in SEQ ID NO: 1 as the primers for PCR, the entire coding region can be amplified.

Synthesis of the oligonucleotides can be performed by an ordinary method such as a phosphoamidite method (see *Tetrahedron Letters*, *22*, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.) using *Taq* DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designate by the supplier.

The *rhtC* gene was obtained in the DNA fragment including *rhtB* gene by chance when *rhtB* was cloned as described later in the embodiments. The *rhtC* gene corresponds to a corrected, as described below, sequence of O128 (nucleotide numbers 60860-61480 of GeneBank

accession number M87049) which is a known but function-unknown ORF. The *rhtC* gene may be obtained by PCR or hybridization using oligonucleotides designed according to the nucleotide sequence of SEQ ID NO: 3. By using olidonucleotides having nucleotide sequence corresponding to a upstream region from nucleotide number 187 and a downstream region from the nucleotide number 804 in SEQ ID NO: 3 as the primers for PCR, the entire coding region can be amplified.

In the present invention, the DNA coding for the RhtB protein of the present invention may code for RhtB protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rh activity of RhtB protein encoded thereby is not deteriorated. Similarly, the DNA coding for the RhtC protein of the present invention may code for RhtC protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rt activity of RhtC protein encoded thereby is not deteriorated.

The DNA, which codes for the substantially same protein as the RhtB protein or RhtC protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-

directed mutagenesis method so that one or more amino acid residues at a specified site involve deletion, substitution, insertion, or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the RhtB protein or RhtC protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium, belonging to the genus *Escherichia* harboring a DNA coding for the RhtB protein with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoquanidine (NTG) and nitrous acid usually used for the mutation treatment.

The DNA, which codes for substantially the same protein as the RhtB protein or RhtC protein, can by obtained by expressing a DNA subjected to *in vitro* mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine or threonine, and selecting the DNA which increase the resistance.

It is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants.

Therefore the DNA, which codes for substantially the

same protein as the RhtC protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 187 to 804 of the nucleotide sequence of SEQ ID NO: 3 or a probe obtainable therefrom under stringent conditions, and which codes for a protein having the Rt activity from a bacterium belonging to the genus *Escherihia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherihia*.

Also, the DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence of SEQ ID NO: 1 or a probe obtainable therefrom under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus *Escherichia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherichia*.

The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any

numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized. Alternatively, the stringent condition is exemplified by a condition under which DNA's are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 60 °C, 1 x SSC, 0.1 % SDS, preferably 0.1 x SSC, 0.1 % SDS.

<2> Bacterium belonging to the genus *Escherichia* of the present invention

The bacterium belonging the genus *Escherichia* of the present invention is a bacterium belonging to the genus *Escherichia* of which the Rt activity is enhanced. Preferred embodiment of the bacterium of the present invention is a bacterium which is further enhanced the Rh activity. A bacterium belonging to the genus *Escherichia* is exemplified by *Escherichia coli*. The Rt activity can be enhanced by, for example, amplification of the copy number of the *rhtC* structural gene in a cell, or transformation of a bacterium belonging to the genus *Escherihia* with a recombinant DNA in which a DNA

fragment including the *rhtC* structural gene encoding the RhtC protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherihia*. The Rt activity can be also enhanced by substitution of the promoter sequence of the *rhtC* gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*.

Besides, the Rh actibity can be enchanced by, for example, amplification of the copy number of the *rhtB* structural gene in a cell, or transformation of a bacterium belonging to the genus *Escherichia* with recombinant DNA in which a DNA fragment including the *rhtB* structural gene encoding RhtB protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*. The Rh activity can be also enhanced by substitution of the promoter sequence of the *rhtB* gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*.

The amplification of the copy number of the *rhtC* structural gene or *rhtB* structural gene in a cell can be performed by introduction of a multicopy vector which carries the *rhtC* structural gene or *rhtB* structural gene into a cell of a bacterium belonging to the genus

Escherihia. Specifically, the copy number can be increased by introduction of a plasmid, a phage or a transposon (Berg, D.E. and Berg, C.M., Bio/Tecnol., 1, 417 (1983)) which carries the *rhtC* structural gene or *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*.

The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as λ1059, λBF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D.A M.Morrison (Methods in Enzymology, 68, 326 (1979)) or a method in which recipient bacterial cell are treated with calcium chloride to increase permeability of DNA (Mandel, M. And Higa, A., J. Mol. Biol., 53, 159, (1970)) and the like.

If the Rt activity, or the Rt activity and the Rh activity is enhanced in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* which is to be the Rt activity, or the Rt activity and the Rh activity is enhanced, strains which

have abilities to produce desired amino acids are used. Besides, an ability to produce an amino acid may be imparted to a bacterium in which the Rt activity, of the Rt activity and Rh activity is enhanced.

On the basis of the rhtC DNA fragment amplification the new strains *E. coli* MG442/pRhtC producing homoserine; *E. coli* MG442/pVIC40,pRhtC producing threonine; *E. coli* NZ10/pRhtBC and *E. coli* NZ10/pRhtB, pRhtC producing homoserine, valine and leucine were obtained which accumulate the amino acids in a higher amount than those containing no amplified rhtC DNA fragment.

The new strains have been deposited (according to international deposition based on Budapest Treaty) in the All-Russian Collection for Industrial Microorganisms (VKPM). The strain *E. coli* MG442/pRhtC has been deposited as an accession number of VKPM B-7700; the strain *E. coli* MG442/pVIC40,pRhtC has been deposited as an accession number of VKPM B-7680; the strain *E. coli* NZ10/pRhtB, pRhtC has been deposited as an accession number of VKPM B-7681, and the strain *E. coli* NZ10/pRhtBC has been deposited as an accession number of VKPM B-7682.

The strain *E. coli* MG442/pRhtC (VKPM B-7700) exhibits the following cultural-morphological and

biochemical features.

Cytomorphology

Gram-negative weakly-motile rods having rounded ends. Longitudinal size, 1.5 to 2 μm.

Cultural features

Beef-extract agar:

After 24 hours of growth at 37° C. produces round whitish semitransparent colonies 1.0 to 3 mm in diameter, featuring a smooth surface, regular or slightly wavy edges, the centre is slightly raised, homogeneous structure, pastelike consistency, readily emulsifiable.

Luria's agar:

After a 24-hour growth at 37° C. develops whitish semitranslucent colonies 1.5 to 2.5 mm in diameter having a smooth surface, homogeneous structure, pastelike consistency, readily emulsifiable.

Minimal agar-doped medium M9:

After 40 to 48 hours of growth at 37°C forms colonies 0.5 to 1.5 mm in diameter, which are coloured greyish-white, semitransparent, slightly convex, with a

lustrous surface.

Growth in a beaf-extract broth:

After a 24-hour growth at 37° C exhibits strong uniform cloudiness, has a characteristic odour.

Physiological and biochemical features.

Grows upon thrust inoculation in a beef-extract agar:

Exhibits good growth throughout the inoculated area. The microorganism proves to be a facultative anaerobe.

It does not liquefy gelatin.

Features a good growth on milk, accompanied by milk coagulation.

Does not produce indole.

Temperature conditions: Grows on beaf-extract broth at 20-42°C, an optimum temperature lying within 33-37 °C.

pH value of culture medium: Grows on liquid media having the pH value from 6 to 8, an optimum value being 7.2.

Carbon sources: Exhibits good growth on glucose, fructose, lactose, mannose, galactose, xylose, glycerol, mannitol to produce an acid and gas.

Nitrogen sources: Assimilates nitrogen in the form of ammonium, nitric acid salts, as well as from some

organic compounds.

Resistant to ampicillin.

L-isoleucine is used as a growth factor. However, the strain can grow slowly without isoleucine.

Content of plasmids: The cells contain multicopy hybrid plasmid pRhtC ensuring resistance to ampicillin (100 mg/l) and carrying the rhtC gene responsible for the increased resistance to threonine (50 mg/ml).

The strain *E. coli* MG442/pVIC40, pRhtC (VKPM B-7680) has the same cultural-morphological and biochemical features as the strain VKPM B-7700 except for in addition to pRhtC, it contains a multicopy hybrid plasmid pVIC40 ensuring resistance to streptomycin (100 mg/l) and carrying the genes of the threonine operon.

The strain *E. coli* strain *E. coli* NZ10/pRhtB, pRhtC (VKPM B-7681) has the same cultural-morphological and biochemical features as the strain VKPM B-7700 except for L-threonine (0.1 - 5 mg/ml) is used as a growth factor instead of L-isoleucine. Besides, it contains a multicopy hybride plasmid pRhtB ensuring resistance to kanamycin (50 mg/l) and carrying the rhtB gene which confers resistance to homoserine (10 mg/ml)

The strain *E. coli* strain *E. coli* NZ10/pRhtBC, (VKPM B-7682) has the same cultural-morphological and biochemical features as the strain VKPM B-7681 except

for it contains a multicopy hybride plasmid pRhtBC ensuring resistance to ampicillin (100 mg/l) and carrying both the rhtB and rhtC genes which confer resistance to L-homoserine (10 mg/ml) and L-threonine (50mg/ml).

<3> Method for producing an amino acid

An amino acid can be efficiently produced by cultivating the bacterium in which the Rt activity, or the Rt activity and Rh activity is enhanced by amplifying a copy number of the *rhtC* gene, or *rhtC* gene and *rhtB* gene as describe above, and which has an ability to produce the amino acid, in a culture medium, producing and accumulating the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-threonine and branched chain amino acids. The branched chain amino acids may be exemplified by L-valine, L-leucine and L-isoleucine, and preferably exemplified by L-valine, L-leucine.

In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acids from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino

acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amount. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium. Alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyzate and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

The cultivation is preferably culture under an aerobic condition such as a shaking, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. the pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the

accumulation of the target amino acid in the medium.

Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

Brief Explanation of Drawings

Fig. 1 shows cloning and identification of rhtB and rhtC genes,

Fig. 2 shows structure of the plasmid pRhtB harboring *rhtB* gene,

Fig. 3 shows structure of the plasmid pRhtC harboring *rhtC* gene, and

Fig. 4 shows structure of the plasmid pRhtBC harboring *rhtB* gene and *rhtC* gene.

Best Mode for Carrying Out the Invention

The present invention will be more concretely explained below with reference to Examples. In the Examples, an amino acid is of L-configuration unless otherwise noted.

Example 1: Obtaining of the rhtB and rhtC DNA fragments

Step 1. Cloning of genes involving resistance homoserine and threonine into mini-Mu phagemid

The genes involving resistance homoserine and threonine were cloned *in vivo* using mini-Mu d5005 phagemid (Groisman, E.A., et al., J. Bacteriol., 168, 357-364 (1986)). MuCts62 lysogen of the strain MG442 (Guayatiner et al., Genetika (in Russian), 14, 947-956 (1978)) was used as a donor. Freshly prepared lysated were used to infect a Mucts lysogenic derivative of a strain VKPM B-513 (Hfr K10 metB). The cells were plated on M9 glucose minimal medium with methionine (50 μg/ml), kanamycin (40 μg/ml) and homoserine (10 μg/ml). Colonies which appeared after 48 hr were picked and isolated. Plasmid DNA was isolated and used to transform the strain VKPM B-513 by standard techniques. Transformants were selected on L-broth agar plates with kanamycun as above. Plasmid DNA was isolated from those which were resistance to homoserine, and analyzed by restriction mapping of the structure of the inserted fragments. It appeared that two types of inserts belonging to different chromosome regions had been cloned from the

donor. Thus, at least two different genes that in multicopy impart resistance to homoserine exist in *E. coli*. One of the two types of inserts is the *rhtA* gene which has already reported (ABSTRACT of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting af the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997). Among the other of the two types of inserts, a MluI-MluI fragment of 0.8 kb imparts only the resistance to homoserine (Fig. 1).

Step 2: Identification of rhtB gene and rhtC gene

The insert fragment was sequenced by the dideoxy chain termination method of Sanger. Both DNA strands were sequenced in their entirety and all junctions were overlapped. The sequencing showed that the insert fragment included f138 (nucleotide numbers 61543 to 61959 of GenBank accession number M87049) which was a known but function-unknown ORF (open reading frame) present at 86 min of *E. coli* chlomosome and about 350 bp of an upstream region thereof (downstream region in the sequence of M87049). The f138 which had only 160 nucleotides in the 5′-flanking region could not impart the resistance to homoserine. No termination codon is

present upstream the ORF f138 between 62160 and 61950 nucleotides of M87049. Furthermore, one ATG following a sequence predicted as a ribosome binding site is present in the sequence. The larger ORF (nucleotide numbers 62160 to 61546) is designated as *rhtB* gene. The RhtB protein deduced from the gene has a region which is highly hydrophobic and contais possible transmembrane segments.

As described below, the plasmid containing this gene conferred upon cells only the resistance to high concentrations of homoserine. Since the initial *Sac*II-*Sac*II DNA fragment contained the second unidentified ORF, 0128, the gene was subcloned and tested for its ability to confer resistance to homoserine and threonine. It proved that the plasmid containing o128 (*Cla*I-*Eco*47III fragment) conferred resistance to 50 mg/ml threonine (Fig. 1). The subcloned fragment was sequenced and found to contain additional nucleotide (G) in the position between 61213 and 61214 nucleotides of M87049. The nucleotide addition to the sequence eliminated a frame shift and enlarged the ORF into 5'-flanking region up to 60860 nucleotide. This new gene was designated as *rhtC*. Both genes, *rhtB* and *rhtC*, were found to be homologous to transporter involved in lysine export of *Corynebacterium glutamicum*.

Example 2: The effect of rhtB and rhtC genes amplification on homoserine production.

<1> Construction of the L-homoserine-producing strain *E. coli* NZ10/pAL4, pRhtB and homoserine production

The *rhtB* gene was inserted to a plasmid pUK21 (Vieira, J. And Messing, J., Gene, 100, 189-194 (1991)), to obtain pRhtB (Fig. 2).

Strain NZ10 of *E. coli* was transformed by a plasmid pAL4 which was a pBR322 vector into which the *thrA* gene coding for aspartokinase-homoseine dehydrogenase I was inserted, to obtain the strains NZ10/pAL4. The strain NZ10 is a *leuB*$^{+}$-reverted mutant *thrB*$^{-}$ obtained from the *E. coli* strain C600 (*thrB*, *leuB*) (Appleyard R.K., Genetics, 39, 440-452,1954).

The strain NZ10/pAL4 was transformed with pUK21 or pRhtB to obtain strains NZ10/pAL4,pUK21 and NZ10/pAL4, pRhtB.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l ampicilin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium having the following composition and containing 50 mg/l kanamycin and 100 mg/l ampicilin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48

hours with a rotary shaker. After the cultivation, an accumulated amount of homoserine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

[Fermentation medium composition (g/L)]

Glucose	80
$(NH_4)_2SO_4$	22
K_2HPO_4	2
NaCl	0.8
$MgSO_4 \cdot 7H_2O$	0.8
$FeSO_4 \cdot 7H_2O$	0.02
$MnSO_4 \cdot 5H_2O$	0.02
Thiamine hydrochloride	0.2
Yeast Extract	1.0
$CaCO_3$	30

($CaCO_3$ was separately sterilized)

The results are shown in Table 1. As shown in Table 1, the strain NZ10/pAL4,pRhtB accumulated homoserine in a larger amount than the strain NZ10/pAL4,pUK21 in which the *rhtB* gene was not enhanced.

Table 1.

Strain	OD_{560}	Accumulated amount of homoserine(g/L)
NZ10/pAL4,pUK21	14.3	3.3
NZ10/pAL4,pRhtB	15.6	6.4

<2> Construction of the homoserine-producing strain *E. coli* MG442/pRhtC and homoserine production

The *rhtC* gene was inserted to pUC21 vector (Vieira, J. And Messing, J., *Gene*, *100*, 189-194 (1991)), to obtain pRhtC (Fig. 3).

The known *E. coli* strain MG442 which can produce threonine in an amount of not less than 3 g/L (Gusyatiner, *et al.*, 1978, Genetika (in Russian), 14:947-956) was transformed by introducing pUC21 or pRhtC to obtain the strains MG442/pUC21 and MG442/pRhtC.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/ml ampicilin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium describe above and containing 100 mg/ml ampicilin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of homoserine in the medium and an absorbance at 560 nm of the medium were determined by known methods. The results are shown in Table 2.

Table 2.

Strain	OD_{560}	Accumulated amount of homoserine (g/L)
MG442/pUC21	9.7	<0.1
MG442/pRhtC	15.2	9.5

Example 3: The effect of rhtB and rhtC genes amplification on threonine production.

<1> Construction of the threonine -producing strain *E. coli* VG442/pVIC40, pRhtB (VKPM B-7660) and threonine production

The strain MG442 was transformed by introducing a known plasmid pVIC40 (U.S. Patent No. 5,175,107 (1992)) by an ordinary transformation method. Transformants were selected on LB agar plates containing 0.1 mg/ml streptomycin. Thus a novel strain MG442/pVIC40 was obtained.

The strain MG442/pVIC40 was transformed with pUK21 or pRhtB to obtain strain MG442/pVIC40,pUK21 and MG442/pVIC40,pRhtB.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l streptomycin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a

fermentation medium describe in Example 2 and containing 50 mg/l kanamycin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 68 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

The results are shown in Table 3. As shown in Table 3, the strain MG442/pVIC40,pRhtB accumulated threonine in a larger amount than the strain MG442/pVIC40,pUK21 in which the *rhtB* gene was not enhanced.

Table 3.

Strain	OD_{560}	Accumulated amount of threonine (g/L)
MG442/pVIC40,pUK21	16.3	12.9
MG442/pVIC40,pRhtB	15.2	16.3

<2> Construction of the threonine-producing strain *E. coli* VG442/pVIC40, pRhtC (VKPM B-7680) and threonine production

The strain MG442/pVIC40 was transformed with pRhtC and pUC21. Thus the transformants MG442/pVIC40,pRhtC and MG442/pVIC40, pUC21 were obtained. In the sane manner as describe above, MG442/pVIC40,pUC21 and

MG442/pVIC40,pRhtC were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicilin and 100 mg/l streptomycin and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium describe above and containing 100 mg/l ampicilin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

The results are shown in Table 4. As shown in Table 4, the strain MG442/pVIC40,pRhtC accumulated threonine in a larger amount than the strain MG442/pVIC40,pUC21 in which the *rhtC* gene was not enhanced.

Table 4

Strain	OD_{560}	Accumulated amount of threonine (g/L)
MG442/pVIC40, pUC21	17.4	4.9
MG442/pVIC40,pRhtC	15.1	10.2

<u>Example 4: Concerted effect of rhtB gene and rhtC gene on amino acid production</u>

The *Sac*II-*Sac*II DNA fragment containing both *rhtB* and rhtC genes was inserted to the pUC21. Thus the plasmid pRhtBC was obtained which harbors the *rhtB* gene

and *rhtC* gene (Fig. 4).

Then, the strain NZ10 was transformed with pUC21, pRhtB, pRhtC or pRhtBC, and the transformants NZ10/pUC21 (VKPM B-7685), NZ10/pRhtB (VKPM B-7683), NZ10/pRhtC (VKPM B-7684), NZ10/pRhtB, pRhtC (VKPM B-7681) and NZ10/pRhtBC (VKPM B-7682) were thus obtained.

The transformants obtained above were cultivated as the same manner as describe above and accumulated amounts of various amino acids in the medium and an absorbance at 540 nm of the medium were determined by known methods.

The result were shown in Table 5. It follows from the Table 5 that concerted effect of the pRhtB and pRhtC on producrion of homoserine, valine and leucine. These results indicate that the *rhtB* and *rhtC* gene products may interact in cells.

Table 5.

Strain	OD_{560}	Homoserine (g/L)	Valine (g/L)	Leucine (g/L)
NZ10/pUC21	18.7	0.6	0.22	0.16
NZ10/pRhtB	19.6	2.3	0.21	0.14
NZ10/pRhtC	20.1	0.7	0.2	0.15
NZ10/pRhtBC	21.8	4.2	0.34	0.44
NZ10/pRhtB,pRhtC	19.2	4.4	0.35	0.45

Example 5: Effect of rhtB gene and rhtC gene on resistance to amino acids

As describe above, the plasmids harboring the *rhtB* and *rhtC* have positive effect on some amino acid accumulation in culture broth by different strains. It proved that the pattern of accumulated amino acid was dependent on the strain genotype. The homology of the *rhtB* and *rhtC* genes products with the lysine transporter LysE of *Corynebacterium glutamicum* (Vrljic, M., Sahm, H. and Eggeling, L. (1996) *Mol. Microbiol. 22*, 815-826.) indicates the analogues function for these proteins.

Therefore, the effect of the pRhtB and pRhtC plasmids on susceptibility of the strain N99 which is a streptomycin-resistant (Str^R) mutant of the known strain W3350 (VKPM B-1557) to some amino acids and amino acid

analogues was tested. Overnight broth cultures (10^9 cfu/ml) of the strains N99/pUC21, N99pUK21, N99/pRhtB and N99/pRhtC were diluted 1:100 in M9 minimal medium and grown for 5 h in the same medium. Then the log phase cultures thus obtained were diluted and about 10^4 viable cells were applied to well-dried test plates with M9 agar (2%) containing doubling increments of amino acids or analogues. Thus the minimum inhibitory concentration (MIC) of these compounds were examined.

The result are shown in Table 6. It follows from the Table 6 that multiple copies of *rhtB* besides homoserine conferred increased resistance to α-amino-β-hydroxyvaleric-acid (AHVA) and S-(2-aminoethyl)-L-cysteine (AEC), and 4-aza-DL-leucine; and multiple copies of *rhtC* gene besides threonine increased resistance to valine, histidine, and AHVA. This results indicates that every of the presumed transporters, *RhtB* and *RhtC*, have specificity to several substrates (amino acids), or may shown non-specific effects as a result of amplification.

Table 6.

Substrate	MIC (μg/ml)		
	N99/pUC21*	N99/pRhtB	N99/pRhtC
L-homoserine	1000	20000	1000
L-threonine	30000	40000	80000
L-valine	0.5	0.5	2.0
L-histidine	5000	5000	40000
AHVA	100	2000	15000
AEC	5	20	5
4-aza-DL-leucine	50	100	50
O-methyl-L-threonine	20	20	20

*: The same data were obtain with N99/pUK21.

Sub B1

SEQUENCE LISTING

<110> Livshits, Vitaliy Arkadyevich
Zakatacva, Natalia Pavlvna
Aleshin, Vladimir Veniaminovich
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<120> NOVEL GENE AND METHOD FOR PRODUCING L-AMINO ACIDS

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Del B1

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